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RESEARCH ARTICLE

Genomic profile of maize response to *Aspergillus flavus* infection

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Abstract

The opportunistic pathogen *Aspergillus flavus* infects important seed crops, including corn, peanuts, and cotton. *A. flavus* is capable of producing mycotoxins called aflatoxins. Aflatoxin B_1, the major mycotoxin contaminant of maize, is a potent carcinogen and has been directly linked to hepatocellular carcinoma. Natural sources of maize fungal resistance exist, but efforts to increase resistance through traditional plant breeding have yielded little success. Using the maize Unigene 1-1.05 arrays, a comparison of resistant (Mp313E) and susceptible (Va35) inbred maize lines 48 hours post-*A. flavus* infection identified 236 genes as significant. During infection, 135 genes were up-regulated in the susceptible maize line Va35, 112 genes were up-regulated in the resistant maize line Mp313E, 12 genes were up-regulated in both lines, and 1 gene was down-regulated in both lines compared to uninfected lines. Comparisons of the biological profile responses of these maize lines revealed a striking difference in reaction to infection. These identified genes will serve as the initial step for developing molecular markers to understand this complex interaction and help with introgression of *A. flavus* resistance into maize hybrids.

Keywords: *Aflatoxin;* Aspergillus flavus*; cDNA microarray; disease resistance; unigene;* Zea mays

Introduction

Aspergillus flavus is an opportunistic pathogen in maize (*Zea mays* L.) and other oilseed crops. During pathogenesis, *Aspergillus* can produce potent mycotoxins called aflatoxins. The most commonly produced aflatoxins are B_1 and B_2, though some isolates also produce G_1 and G_2 (Geiser et al., 2000). Aflatoxin B_1 is the form most frequently found in infections of maize. Aflatoxins are highly toxic, hepatocarcinogenic, and mutagenic (Bressac et al., 1991; Hsu et al., 1991; Wogan, 1992). Concentrations as low as 0.05 μg ml^{-1} have been shown to totally inhibit the growth of human embryonic lung cells (Legator et al., 1965). Due to the health effects of aflatoxins, the FDA enforces a 20 ppb limit for human consumption, while a 2 ppb limit exists in the European Union (Mahoney and Molyneux, 2004). These restrictions directly result in over $250 million of lost maize-related revenues each year in the United States (Richard and Payne, 2003; Vardon et al., 2003). In an effort to reduce these economic and health impacts, multiple studies of *Aspergillus*, aflatoxins, and maize resistance have occurred (Abbas et al., 2002; Bhatnagar et al., 2003, 2004; Windham and Williams, 2002 Yu et al., 2002, 2004). From these efforts, a number of maize lines resistant to aflatoxin have been developed and released (Williams, 2006; Williams and Windham, 2006).

Resistant lines show a significant decrease in aflatoxin accumulation when compared with susceptible lines (Abbas et al., 2002; Bhatnagar et al., 2004; Williams, 2006; Windham and Williams, 2002; Wicklow,

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1983). Although resistant, once *A. flavus* is introduced into the ear, the conidia germinate and can produce aflatoxin (Magbanua, 2004). Additionally, *A. flavus* conidia can germinate on the silk surfaces of maize, directly progressing to the glumes and finally colonizing the kernel (Marsh and Payne, 1984). However, very little information is available on the progress of the fungus within the maize tissues after inoculation.

Microscopic examination of wound-inoculated susceptible ears demonstrates that the fungus spreads from the wound and by 28 days postinoculation can be found throughout all rachis tissues (Smart et al., 1990). Similarly, inoculation of developing maize ears 20 days after silk emergence with a green fluorescent (GFP)–tagged *A. flavus* showed significantly higher fluorescence in the pith of susceptible maize hybrids than in the pith of resistant hybrid lines; this difference was detectable as soon as 24 hours after inoculation (Magbanua, 2004). In the susceptible lines, earlier inoculation time points exhibited the highest fluorescence, indicating ear age is critical in resistance. GFP fluorescence was also visible in the resistant lines but at low levels, and the level of fluorescence was almost always consistent over time. This observation may indicate that the fungus is sustained even in these lines, but its growth is arrested by resistance factors in the ear and, in particular, the rachis (Magbanua, 2004).

When Mp313E, a particularly promising resistant inbred line, is used to generate maize hybrids, aflatoxin resistance is consistently inherited. Additional studies have shown resistant alleles also originate from the susceptible parents (Davis and Williams, 1999). Thus, with resistance alleles existing in both resistant and susceptible lines, it is necessary to examine both parental lines to identify contributions to resistance.

Genetic studies on the descendants of the resistant Mp313E crossed to susceptible Va35 line have identified a number of chromosomal regions associated with reduced aflatoxin accumulation, termed quantitative trait loci (QTL) (Brooks et al., 2005; Busboom and White, 2004; Davis and Williams, 1999). Up to 48% of Mp313E's resistance can be assigned to the QTL located on chromosomes 2, 3, and 4 (Brooks et al., 2005; Warburton et al., 2009). Unfortunately, efforts to increase host resistance of production lines (elite lines) by integrating resistance found on these QTL through traditional plant breeding have yielded little success. Failure to integrate resistance has been due to the lack of sufficiently accurate markers to track resistance during crossing and selection of hybrid lines. Using QTL to identify individual candidate genes is difficult due to complications introduced by environmental factors (Wayne and McIntyre, 2002).

High temperatures, drought, and physical injuries are conducive to *A. flavus* infection and aflatoxin contamination in maize (Luo et al., 2005; Magbanua, 2004; Widstrom et al., 2003;). However, the variability of these effects confounds most field evaluations making QTL analysis, aflatoxin concentration determination, and infection rates erratic and difficult to interpret. The end result is that evaluation from year to year, location to location, and time point to time point can show large degrees of variability (Widstrom et al., 2003).

Microarrays are capable of taking a snapshot of an organism's response and thus yield accurate information despite the variation of environmental parameters. By utilizing maize microarrays, it is possible to couple the gene expression of resistant and susceptible lines to previously established QTL maps to yield a more precise identification of aflatoxin resistance genes. To that end, we report the expression profiles for resistance to *A. flavus* in the susceptible maize inbred Va35 and resistant maize inbred Mp313E lines during infection with *A. flavus* NRRL 3357.

Materials and methods

Plant materials and experimental design

The maize inbred lines Va35 and Mp313E were selected for this study. Maize line Va35 has yellow kernels and is susceptible to infection by *A. flavus* (Henderson, 1976). Mp313E is a white dent inbred line and was released primarily as a source of resistance to kernel infection by *A. flavus* (Scott and Zummo, 1990). Seeds of Va35 and Mp313E are maintained by the U.S. Department of Agriculture, Agricultural Research Service, Corn Host Plant Resistance Research Unit (USDA-ARS-CHPRRU) at the R. R. Foil Plant Science Farm, Mississippi State, Mississippi. The field experimental design was a randomized complete block arranged as a split plot with three replications. The treatment design was a 2×2 factorial with the two genotypes and two inoculation treatments (inoculated and uninoculated). Genotypes were planted in main plots and inoculation treatments were applied to subplots. Rows were 4 meters long and spaced 0.97 meters apart with a fallow alley of 1 meter. Primary ears on all plants in all plots were self-pollinated by hand. Plots received supplemental furrow irrigation throughout the growing season to mitigate drought stress. Herbicides and fertilizer (application based on soil tests) were applied according to standard cultural practices in corn for a continuous production system in northern Mississippi.

Fungal material

A. flavus isolate NRRL 3357 (ATCC # 200026; SRRC 167), a wild-type strain widely used in laboratory and field studies and known to produce high levels of aflatoxin in corn grain (Windham and Williams, 2002), was chosen for this study. Cultures were grown on sterile corncob grits in 500-ml flasks, each containing 50 g of grits and 100 ml of H_2O, and incubated at 28°C for 3 weeks. Conidia were washed from the grits using 500 ml sterile distilled water containing 0.02% (vol vol^{-1}) Tween 20 (Sigma-Aldrich Co., St. Louis, MO, USA) (polyoxyethylene [20] sorbitol monolaurate) and filtered through four layers of sterile cheesecloth. Conidial concentrations were determined by hemacytometer and adjusted with sterile distilled water to 9×10^7 conidia ml^{-1}. Inoculum not used immediately was refrigerated at 4°C. All maize lines were inoculated 14 days after pollination (DAP) using the side-needle technique (Zummo and Scott, 1989). Plants in inoculated plots were injected between the husks and the kernels with 3.4 ml of a suspension containing approximately 3×10^8 spores ml^{-1} of *A. flavus* conidia. Typically, two to three kernels are damaged by this inoculation technique.

Tissue collection

For each line, two inoculated and two uninoculated primary ears were harvested by hand from each replicate 16 DAP (2 days after inoculation). Harvested ears were maintained on ice until sampling of an approximately 1 cm cross-sectional portion of kernel and cob tissue at the inoculation site, typically found at the mid-section of the ear. Samples were flash frozen in liquid nitrogen, homogenized in a chilled mortar and pestle, sub-sampled into 1-gram sections, and stored at −80°C.

Aflatoxin accumulation analysis

All remaining primary ears from the field were harvested 60 days after the inoculation (DAI) and dried at 38°C for 7 days in a forced air oven. Ears from each replication were shelled, and the grain was thoroughly mixed before grinding in a Romer Series II Mill (Romer Labs, Union, Missouri). Aflatoxin analyses were performed on 50 g subsamples using the Vicam Aflatest (Vicam, Watertown, Massachusetts), as previously described by Windham and Williams (1998). To stabilize variances, the aflatoxin data were log-transformed, and the geometric means (antilogarithm of the logarithmic mean) for aflatoxin accumulation in both Va35 and Mp313E for the inoculated and uninoculated samples were determined.

RNA Isolation and preparation of poly (A^+) RNA + mRNA using Dynabeads oligo $(dT)_{25}$-Dynal

A 1-gram sample of powdered tissue was combined with 10 ml of TRIZOL reagent (Invitrogen, Carlsbad, California) and homogenized briefly with mortar and pestle. Total RNA was extracted as per manufacturer's instructions. Isolated total RNAs were treated with DNase I (Qiagen, Valencia, California) before purification using the RNeasy MinElute Column (Qiagen, Valencia, California). The purity and integrity of the total RNA was checked by running 1% agarose gel electrophoresis and by using a UV/Visible spectrophotometer.

Highly purified, intact mRNA was isolated from total RNA from each sample with Dynabeads® Oligo $(dT)_{25}$-Dynal (Dynal, Oslo, Norway). Dynabeads were prepared as per manufacturer's instructions. A 150 µl aliquot of Dynabeads suspension was added to a new RNase-free micro-centrifuge tube per sample, placed in a magnetic stand and the liquid fraction in all tubes was removed. Following manufacturer's instructions, mRNA was isolated from the total RNA and stored at −80°C until used for labeling.

Isolated mRNAs were used for the preparation of Cy3 labeled and Cy5 labeled cDNA probes. Reverse transcription (RT) PCR (Invitrogen, Carlsbad, California) was used with random hexamer primers to generate cDNAs from the mRNA templates as per manufacturer's instructions. The labeled cDNAs were collected and used immediately for hybridization.

Unigene 1-1.05 arrays

The maize Unigene 1-1.05 arrays purchased from the National Science Foundation (NSF) Maize Gene Discovery Project (MGDP) were selected for this experiment. The Unigene (Pontius et al., 2003) 1-1.05 array slides contain 5,065 expressed sequence tags (EST) contigs from libraries derived from immature leaf, endosperm, immature ear, and the root of maize. These ESTs represent approximately 4,000 genes. To correct for variation, four independent hybridizations were conducted for each maize line.

cDNA hybridization mixture

For hybridization, 40 µl of the cDNA mixture (20 µl of each label), 3 µl Liquid Block™ (Amersham Pharmacia

Biotech, Piscataway, New Jersey), 5 µl 20X SSC, and 2 µl 2% (wt vol^{-1}) sodium dodecyl sulfate (SDS) were combined, denatured at 95°C for 2 min, and immediately transferred to ice. Array slides were denatured at 65°C for 30 seconds before application of hybridization mixture. Slides were then covered with hybrislips (Sigma-Aldrich, St. Louis, Missouri), transferred to a prewarmed hybridization oven (Fisher Scientific, Pittsburg, Pennsylvania), and incubated overnight at 60°C. The hybridized slides were washed on an orbital shaker using 2X SSC, 0.5% (wt vol^{-1}) SDS for 5 min; 0.5X SSC for 5 min; and 0.05X SSC for 5 min, spun dry at low speed in a centrifuge (100×g) for 5 min and scanned using a GenePix Personal 4100A (Molecular Devices Corporation, Union City, California). Spot intensities were determined using GenePix Pro (Livesey et al., 2004) and normalized by adjusting Cy5/Cy3 ratio across all features to 1.0. This is a common normalization strategy that assumes RNAs that deviate up or down from the ratio of 1.0 will balance (Hegde et al., 2000).

cDNA microarray experimental design

The microarray experimental design was a randomized complete block with three replications. From each replication and each genotype in the field, 4 samples (2 inoculated and 2 uninoculated) were collected for a total of 3×4=12 samples. Six slides were used for the 12 samples, with each slide containing the inoculated and uninoculated samples of each genotype. A second slide for each genotype from one replication (rep 2 in the field) of each genotype contained a dye swap. Each contig was represented three times on each slide. Each experiment was repeated two times with independent microarray slides to confirm the reproducibility of the analysis. All recommendations of the minimum requirements for a microarray experiment (MIAME) checklist (Brazma et al., 2001) were observed, and the cDNA microarray data have been deposited in the Gene Expression Omnibus (GSE9546).

Statistical analysis

All data were analyzed with SAS Version 9.1.3 (SAS, Cary, North Carolina). The null hypothesis was defined as follows:

$$H_0: \frac{(\text{Resistant inoculated expression})}{(\text{Resistant uninoculated expression})} = \frac{(\text{Susceptible inoculated expression})}{(\text{Susceptible uninoculated expression})}$$

The median expression level for each spot intensity was log transformed and analyzed by analysis of variance for a split plot design. The main unit was genotype and the subunit treatment was inoculation. Dye was treated as a fixed effect in the model to account for differences in dyes. Therefore, Genotype, Inoculation Treatment, Genotype x Inoculation, Treatment, and Dye were fixed effects in the analysis. Rep (replication), Genotype x Rep, Spots (Rep Genotype), Rep x Inoculation, Treatment (Genotype), and residual subsampling error were the components of error in the analysis. F-test for Genotype x Inoculation Treatment interaction was used to address the null hypothesis shown in the above equation. The mathematical notation for Genotype x Inoculation treatment was H_0: $\text{Log}(Y_{R,I}) - \text{Log}(Y_{R,UI}) = \text{Log}(Y_{S,I}) - \text{Log}(Y_{S,UI})$, where Y = "Yes, expressed gene," and R, S, I, and UI refer to resistant, susceptible, inoculated, and uninoculated, respectively.

Gene ontology annotation of differentially expressed transcripts

Tools from AgBase (www.agbase.msstate.edu) were used for gene ontology (GO) annotation of the differentially expressed transcripts (McCarthy et al., 2006). Parent sequences of the transcripts were used as inputs for the GOanna tool. This tool performs a Blastx comparison of each sequence against the AgBase database. All GO terms for the three highest scoring matches with an E-value less than e-10 were retrieved. All alignments were manually inspected for quality and GO terms were assigned for the highest quality matches. The AgBase GOSlimViewer tool was used to produce high-level summaries of the annotations using the Plant Go Slim developed by Suparna Mundodi and available from the Gene Ontology Web site
(http://www.geneontology.org/GO.slims.shtml).

qRT-PCR validation

To validate the expression level of genes obtained by cDNA microarrays, real-time RT-PCR (Pfaffl, 2001) was conducted for several genes that were significantly expressed. The real-time PCR was conducted on a Roche LightCycler 2.0 instrument. These genes included Beta 5 tubulin chain (AW244904) and NEDD8-like protein RUB2 precursor (AW257929), and the Ubiquitin gene was used as the standard control. High-pressure liquid chromatography (HPLC)-purified oligonucleotide primers were obtained

from Invitrogen (Carlsbad, California). The primer sequences were: Ubiquitin (reference gene) forward primer, 5'-AGCGGGCGACAAATATTCGAGAAC-3' and reverse primer, 5'-TCGAGAACGGAGGTAGT-ACAATGT-3'; Beta 5 tubulin chain (AW244904) forward primer, 5'-GCCTTAGATCCAGCCATGAG-3' and reverse primer, 5'-TCAAGCACGGAGTCAATGAG-3'; NEDD8-like protein RUB2 precursor (AW257929) forward primer, 5'-ACTCCCTTCGGTCTTGAGG-3' and reverse primer, 5'-GCTTCCCAGCATAAAGAGC-3'. The protocol for cDNA preparation from the total RNA, the LightCycler 2.0 PCR run, calibration, calculation and agarose gel electrophoresis testing of the PCR product was from Harfouche et al. (2006).

Results

Aflatoxin accumulation

Aflatoxin levels were determined for infected and uninfected samples collected from both Va35 and Mp313E at 63 days after silking. The uninoculated Va35 and Mp313E had baseline levels at or near Vicam detection levels (approximately 1 ppb). Infected Mp313E increased to 67 ppb and infected Va35 increased to 1,587 ppb, a 24-fold higher accumulation in the susceptible maize line Va35 than in the resistant line Mp313E. Our results are in agreement with those reported by Windham and Williams (2002), who also quantified aflatoxin accumulation in the same inbred lines under field conditions. These differences were based on each genotype tested against the control.

Differential expression and biological grouping

Of the 4,000 genes represented on the arrays, 236 were found to be significantly up-regulated in response to *A. flavus* inoculation ($P < 0.05$) (Tables 1 and 2). A total of 135 of these genes were up-regulated in the susceptible line Va35, 2 showed no change, and 99 were down-regulated. Of the 236 genes that were up-regulated, 112 were up-regulated in the resistant line Mp313E, and 124 were down-regulated. The two lines had 12 genes in common that were up-regulated and

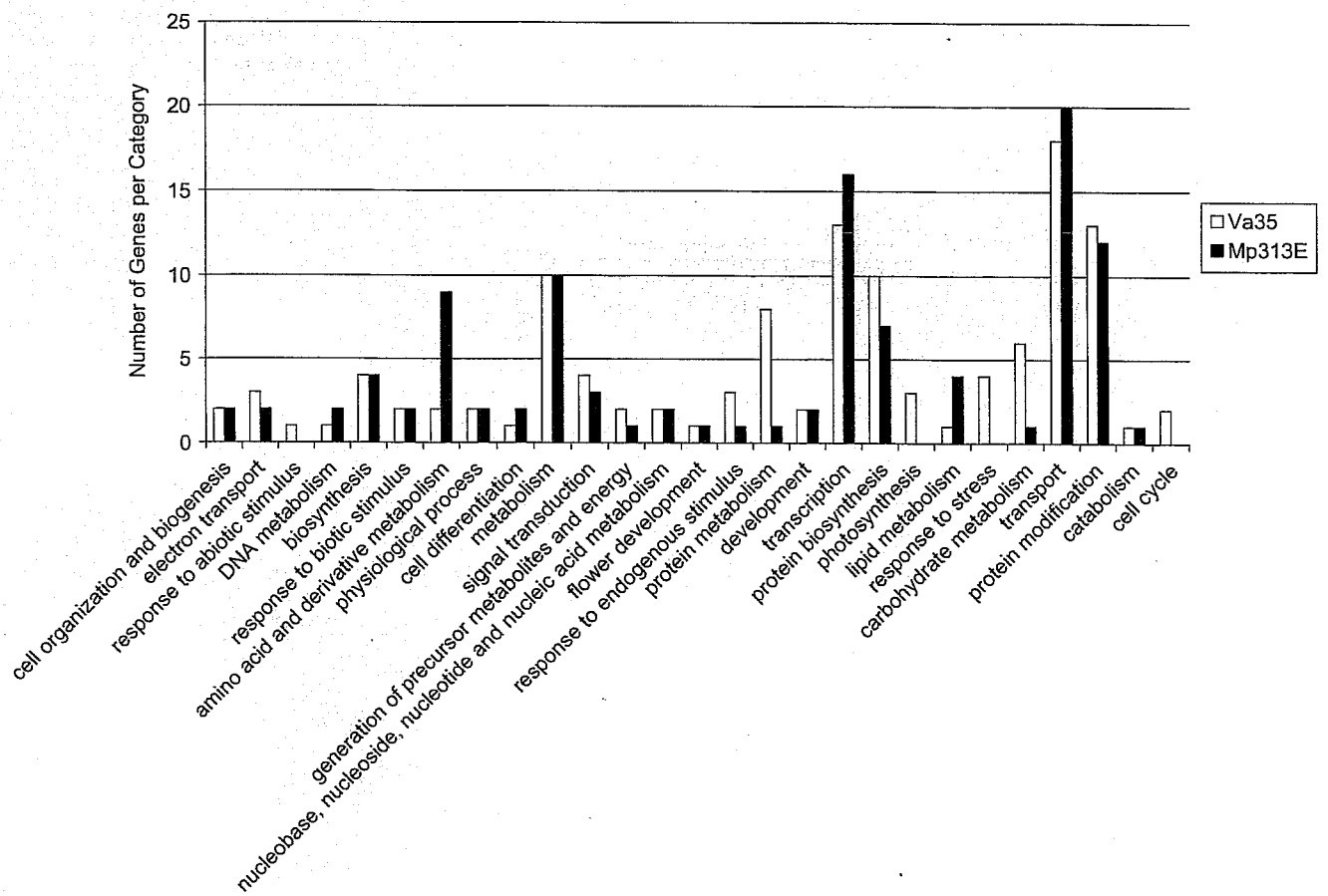


Figure 1. GO biological process classification of genes associated with *A. flavus* resistance identified from cDNA microarray experiment for susceptible (Va35) and resistant (Mp313E) maize lines after 48 hours infection with *A. flavus* NRRL 3357.

1 gene that was down-regulated in both lines. As seen in Figure 1, the up-regulated genes were classified by biological process and divided into several subcategories of functional genes.

Identification of genes associated with A. flavus resistance and QTL mapping

Five specific QTL regions, located on chromosomes 1, 2, 3, 4, and 6, have been identified as correlated

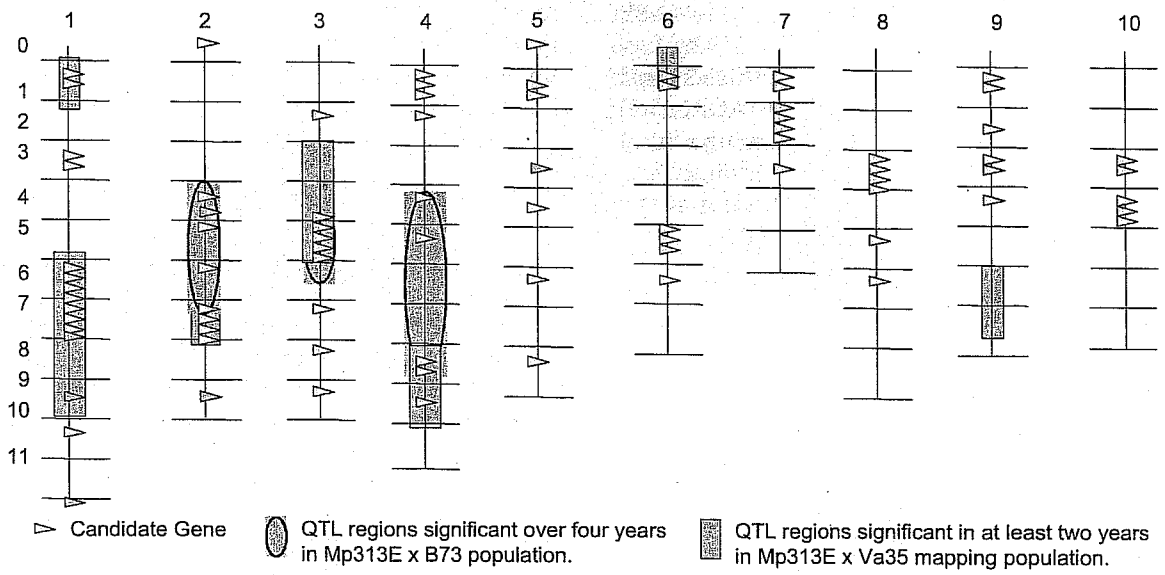


Figure 2. Chromosomal map locations for genes and QTL identified as important to aflatoxin resistance. Divisions on left of figure correspond to chromosomal bin numbers (corresponding individual candidate genes within QTL are detailed in Table 1). QTL regions are defined as in Brooks et al. (2005) and Brooks (unpublished data).

Table 1. List of significantly differentially expressed genes and chromosomal locations.[z]

	Ratio:Inoc Y/N		Genotype*Inoc			
Accession #[y]	MP	VA	FValue	ProbF	Putative Function	Chromosome and Bin Number[x]
AW352524	1.22	0.81	7.7	0.0391	Putative membrane protein	1.01
AW244904	0.71	1.27	11.8	0.0187	Tubulin beta-5 chain	1.01
AW424439	0.80	1.38	18.7	0.0075	Photosystem II 10 kDa polypeptide, chloroplast precursor	1.03
AW257936	0.72	1.28	13.0	0.0154	Mitochondrial import receptor-like protein	1.03
AW433397	1.28	0.78	11.2	0.0204	*No Annotation Assigned*	1.06
AW257929	1.21	1.05	8.3	0.0344	NEDD8-like protein RUB2 precursor	1.06
AI947748	0.88	1.13	8.5	0.0331	*No Annotation Assigned*	1.06
BE012262	0.80	1.02	14.3	0.0129	Oligopeptide transporter 6	1.06/9.03
AW438153	1.34	0.68	79.2	0.0003	DRE binding factor 1	1.07
AW330813	0.82	1.22	9.4	0.0277	*No Annotation Assigned*	1.07
AW061926	0.65	1.43	12.1	0.0177	Beta 1,3-glycosyltransferase-like protein I	1.07
AW331482	0.85	1.32	22.7	0.0050	Chlorophyll a-b binding protein 4, chloroplast precursor	1.07/1.08
AW066119	0.64	1.28	7.1	0.0450	Chaperonin CPN60-like 2, mitochondrial precursor	1.09
AW191159	0.86	1.26	10.2	0.0241	Mitochondrial import receptor subunit TOM20	1.1
BE128894	1.27	0.68	33.2	0.0022	Membrane bound O-acyl transferase-like, tryptophan biosynthesis	1.12
AW061709	0.96	1.29	11.7	0.0189	OSJNBa0018M05.18 protein	2
AW261292	0.88	1.62	15.6	0.0108	Endochitinase B precursor	2.04
AW258084	0.70	1.72	8.4	0.0336	TRANSPORT INHIBITOR RESPONSE 1 protein	2.04/10.04
AW681281	0.73	1.32	8.3	0.0345	*No Annotation Assigned*	2.05
BE055909	2.39	1.05	9.9	0.0256	OSJNBa0009K15.20 protein	2.06/7.02

Table 1. continued on next page

Table 1. Continued.

Accession #[y]	Ratio:Inoc Y/N MP	Ratio:Inoc Y/N VA	Genotype*Inoc FValue	Genotype*Inoc ProbF	Putative Function	Chromosome and Bin Number[z]
AI861230	0.64	1.21	13.0	0.0155	*No Annotation Assigned*	2.07
AW147172	0.87	1.30	10.5	0.0228	Protein disulfide-isomerase precursor	2.09
AW256160	0.74	1.74	11.0	0.0211	60 kDa jasmonate-induced protein	3.02
AW433410	1.02	1.42	27.9	0.0032	Leucine Rich Repeat family protein, expressed	3.05
AW360565	1.20	0.85	21.6	0.0056	Acyl-[acyl-carrier-protein] desaturase, chloroplast precursor	3.05
AW331008	1.20	0.85	9.6	0.0268	*No Annotation Assigned*	3.05
AI948351	0.73	1.15	8.0	0.0366	Probable glutathione S-transferase	3.05
BE128880	1.20	0.78	10.8	0.0219	P0648C09.20 protein	3.05/8.06
AW424498	0.93	1.41	9.0	0.0300	Glucose-1-phosphate adenylyltransferase large subunit 1, chloroplast precursor	3.07
AW216267	0.83	1.13	8.6	0.0328	Naphthoate synthase	3.08
AW244196	0.97	2.39	8.9	0.0306	Glucose-1-phosphate adenylyltransferase large subunit 1, chloroplast precursor	3.09
AW455677	1.16	0.88	10.9	0.0215	Ribulose bisphosphate carboxylase/oxygenase activase, chloroplast precursor	4.01
AW399840	0.85	1.13	8.1	0.0361	*No Annotation Assigned*	4.01
BE056824	1.20	0.76	21.0	0.0059	*No Annotation Assigned*	4.01/4.02
AW433424	0.94	1.71	9.9	0.0253	*No Annotation Assigned*	4.01/4.02
AW231645	0.81	1.30	11.6	0.0193	Zein-alpha GZ19AB11 precursor	4.04
AW225099	0.72	1.10	9.4	0.0280	Leucine-rich repeat receptor protein kinase EXS precursor	4.05
BE129569	1.04	0.69	15.2	0.0114	Chalcone synthase C2	4.08
AW400067	1.12	1.34	7.9	0.0375	Phosphoribosylformylglycinamidine cyclo-ligase, chloroplast precursor	4.08/6.05
AW261420	0.53	1.46	24.7	0.0042	Formate dehydrogenase, mitochondrial precursor	4.09/8.05/ 9.03
AI649566	1.18	0.80	9.8	0.0261	*No Annotation Assigned NCBI putative function DANA2**	5
BE128808	1.33	1.03	8.2	0.0350	Putative GLE1L protein	5.01
AW330755	1.41	1.06	8.2	0.0356	Opaque2 heterodimerizing protein 2	5.01
AW330570	0.78	1.23	38.5	0.0016	Water-stress inducible protein	5.03
AW355894	0.86	1.25	18.3	0.0078	Chlorophyll a-b binding protein M9, chloroplast precursor	5.04
AW461037	1.17	0.69	45.9	0.0011	Membrane bound O-acyl transferase-like	5.06
AW585298	1.09	0.81	10.3	0.0238	Hypothetical protein OJ1695_D07.18	5.08
AW331180	1.88	0.86	9.0	0.0303	Phytoene synthase, chloroplast precursor	6.01
AW144932	1.57	0.73	22.9	0.0050	Actin-depolymerizing factor 6	6.01
BE025386	1.08	0.41	8.4	0.0340	*No Annotation Assigned*	6.05
AW330938	0.76	1.12	10.7	0.0223	*No Annotation Assigned*	6.05
AW231502	0.85	1.40	8.7	0.0321	*No Annotation Assigned*	6.06
BE056994	0.82	1.23	7.9	0.0378	Putative family II extracellular lipase 1	7.01
AW600656	0.78	1.31	23.9	0.0045	*No Annotation Assigned*	7.01
AW400091	1.17	0.86	8.7	0.0317	Spermidine synthase 1	7.02
AW216051	0.62	1.25	32.0	0.0024	*No Annotation Assigned*	7.02
AW179525	0.72	1.25	8.8	0.0310	Cellulose synthase A catalytic subunit 4 [UDP-forming]	7.02
AI948338	0.71	1.07	11.5	0.0195	*No Annotation Assigned*	7.03
BE056107	0.90	1.15	9.3	0.0286	Actin	8.03
BE056070	0.86	1.17	8.2	0.0351	Putative CBL-interacting protein kinase 2	8.03
AW447883	1.24	0.73	21.2	0.0058	*No Annotation Assigned NCBI putative function carnitine/acycarnitine**	8.03
AW147055	0.90	1.35	10.0	0.0251	*No Annotation Assigned*	8.03
AW399895	1.18	0.81	11.2	0.0203	*No Annotation Assigned*	9.01
AW066264	0.52	1.45	10.4	0.0233	*No Annotation Assigned*	9.01
BE055954	1.05	1.37	15.1	0.0116	Putative bZIP transcription factor	9.04
AW927391	1.11	0.72	13.0	0.0154	Putative fasciclin-like arabinogalactan-protein	10.03

Table 1. continued on next page

Table 1. Continued.

Accession #[y]	Ratio:Inoc Y/N MP	VA	Genotype*Inoc FValue	ProbF	Putative Function	Chromosome and Bin Number[x]
AW256192	1.34	0.90	10.7	0.0222	*No Annotation Assigned*	10.04
AW231540	1.31	0.90	23.6	0.0046	OSJNBa0016O02.10 protein	10.04

x Indicates chromosome location as displayed in Fig. 2.
y NCBI accession number (http://www.ncbi.nlm.nig.gov/).
z Statistically significant at $p < 0.05$.

Table 2. Supplementary list of significantly differentially expressed genes[z]

NCBI[y] Accession #	Ratio:Inoc Y/N MP	VA	Genotype*Inoc FValue	ProbF	NCBI[y] Accession #	Ratio:Inoc Y/N MP	VA	Genotype*Inoc FValue	ProbF
AW455735	0.72	1.69	38.3	0.0016	AW400016	1.44	0.90	12.2	0.0173
AW330603	0.76	1.36	34.3	0.0021	AW455616	0.86	1.25	11.9	0.0184
AW787611	1.18	0.74	33.3	0.0022	AW507027	0.65	1.48	11.8	0.0186
AW399955	1.21	0.73	29.3	0.0029	AW455736	0.84	1.40	11.8	0.0187
BE012243	1.14	0.64	28.3	0.0031	VC10	0.81	1.60	11.6	0.0192
AW787410	1.08	0.63	28.2	0.0032	AW225319	1.25	0.83	11.4	0.0196
AW424718	1.13	0.75	25.2	0.0040	AW461025	0.78	1.57	11.4	0.0196
AW261349	1.63	0.68	23.9	0.0045	AW330745	0.73	1.23	11.3	0.0200
AW330586	1.25	0.83	23.6	0.0046	AW787670	1.19	0.68	11.1	0.0209
AW927398	1.27	0.66	22.9	0.0050	AW585293	1.13	0.75	11.0	0.0212
AW585277	0.74	1.21	21.9	0.0055	AW400328	1.68	0.78	10.8	0.0219
AW330811	1.35	0.89	21.4	0.0057	AW433409	1.17	0.80	10.7	0.0220
AW438269	2.14	0.98	21.1	0.0059	AW355987	1.52	0.86	10.6	0.0227
BE056892	1.18	0.75	20.4	0.0063	BE056279	1.15	0.68	10.5	0.0228
AI964629	0.85	1.61	20.0	0.0066	AW129888	0.94	1.31	10.5	0.0230
AW927389	1.22	0.79	20.0	0.0066	AW231611	0.68	1.37	10.3	0.0237
AW313312	0.77	1.16	19.9	0.0067	AI947777	0.67	1.21	10.2	0.0240
AW231313	1.30	0.89	19.8	0.0067	AW330850	1.24	0.91	10.2	0.0240
BE123221	1.08	0.82	19.0	0.0073	AI861119	0.73	1.32	10.2	0.0240
AW927726	1.23	0.74	18.6	0.0077	AW585284	1.30	0.68	10.0	0.0249
AW256149	0.72	1.33	18.3	0.0079	AW331400	0.70	1.45	10.0	0.0249
AW455693	1.27	0.75	18.3	0.0079	AW225165	0.55	1.66	9.9	0.0254
AW355966	0.85	1.25	17.8	0.0084	AW258058	0.70	1.47	9.9	0.0255
AW787650	0.72	1.11	16.9	0.0092	AW424674	0.88	2.05	9.8	0.0257
AW330818	0.84	1.08	16.9	0.0093	PAC 1-3	0.75	1.85	9.8	0.0258
AW399983	1.20	0.81	16.6	0.0096	AW225224	0.72	1.81	9.8	0.0260
BE056054	0.95	1.58	16.3	0.0100	BE057015	1.35	0.92	9.8	0.0261
AW331268	0.81	1.43	16.2	0.0101	AW330585	1.28	0.82	9.7	0.0264
AW447878	1.20	0.73	16.0	0.0103	AW330774	1.14	0.86	9.7	0.0264
BE128793	1.34	0.93	15.9	0.0105	AW191128	0.72	1.17	9.7	0.0265
AW191099	0.96	2.22	15.3	0.0112	AW447841	0.96	1.38	9.6	0.0271
AW424761	1.15	0.75	15.3	0.0113	AW261233	1.23	0.79	9.5	0.0276
AW331478	1.26	0.88	15.3	0.0113	AW257975	1.18	0.78	9.4	0.0281
AW261244	0.67	1.64	15.0	0.0117	AW244911	0.67	1.30	9.3	0.0282
AW352513	1.26	0.78	14.7	0.0121	AW461098	1.10	0.79	9.2	0.0289
BE055960	1.24	0.68	14.6	0.0123	AW065983	0.79	1.19	9.2	0.0290
AW461134	1.37	0.72	14.6	0.0124	AW225216	1.40	1.02	9.2	0.0290
AW927842	1.10	0.64	14.6	0.0124	17A1	0.89	1.42	9.1	0.0295
AW400394	1.15	0.78	14.3	0.0129	AW928268	0.85	1.32	9.0	0.0299
AW331163	0.71	1.17	14.2	0.0130	AW400227	1.05	0.82	9.0	0.0303
AI855427	0.71	1.38	14.1	0.0132	AW461156	1.32	0.76	8.6	0.0324

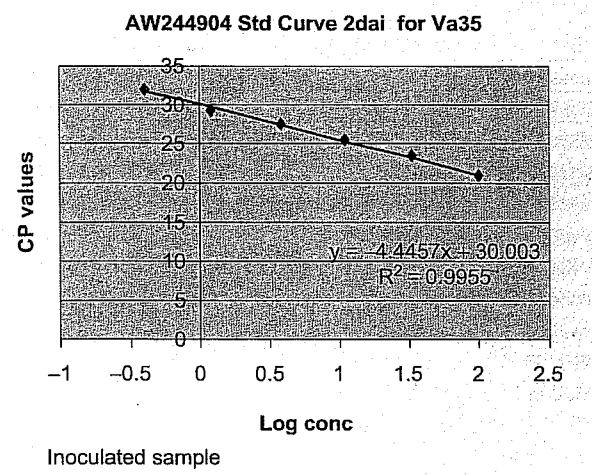
Table 2. continued on next page

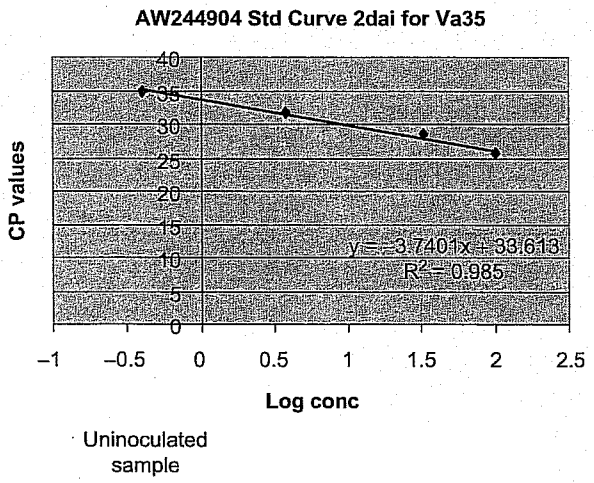
Table 2. Continued.

NCBI[y]	Ratio:Inoc Y/N		Genotype*Inoc		NCBI[y]	Ratio:Inoc Y/N		Genotype*Inoc	
Accession #	MP	VA	FValue	ProbF	Accession #	MP	VA	FValue	ProbF
BE123293	1.13	0.70	13.7	0.0139	AW399897	0.74	1.45	8.6	0.0326
AI861092	1.44	1.00	13.3	0.0149	AW330996	1.38	0.95	8.5	0.0333
AW330873	0.72	1.28	13.0	0.0154	AW224869	1.43	1.01	8.5	0.0335
AW927551	1.39	0.73	12.9	0.0157	AW927386	1.04	0.73	8.4	0.0337
AW231890	0.83	1.56	12.4	0.0169	AW400023	1.15	0.79	8.3	0.0344
BE056240	1.15	0.76	8.2	0.0351	AW424482	0.76	1.08	7.4	0.0415
AW313335	0.64	1.28	8.2	0.0355	AW060056	0.94	2.00	7.4	0.0417
AW400216	1.10	0.72	8.2	0.0355	AW399957	0.80	1.21	7.3	0.0426
AW433443	0.73	1.11	8.1	0.0360	AI947757	0.90	1.55	7.3	0.0429
AW061703	0.66	1.23	8.0	0.0364	AW258116	0.83	1.85	7.3	0.0430
BE129843	1.02	0.74	8.0	0.0365	AI857211	1.35	0.97	7.3	0.0431
AW461038	0.94	1.48	8.0	0.0365	AW225056	0.79	1.14	7.2	0.0432
AW261261	0.77	1.43	8.0	0.0365	AW330684	0.79	1.27	7.2	0.0435
AW360406	0.76	1.58	8.0	0.0368	BE129611	1.11	0.81	7.1	0.0444
AI861259	0.75	1.24	7.9	0.0373	AW360444	0.70	1.16	7.1	0.0449
AW461002	1.36	0.82	7.9	0.0373	BE128815	0.83	1.28	7.1	0.0450
AW400366	1.59	0.70	7.9	0.0376	AW061940	0.84	1.23	7.1	0.0450
AW256258	0.65	1.63	7.9	0.0377	AW066119	0.64	1.28	7.1	0.0450
AW324620	1.31	1.00	7.9	0.0379	BE012213	1.09	0.72	7.0	0.0452
AW424633	1.26	0.71	7.8	0.0380	AW330902	1.21	0.81	7.0	0.0453
AW438394	0.76	1.11	7.8	0.0380	BE056090	0.75	1.07	7.0	0.0453
AW288761	1.61	0.92	7.8	0.0386	AW447856	1.12	0.88	7.0	0.0455
AW231870	1.34	0.96	7.8	0.0387	AW330882	0.66	1.39	7.0	0.0457
BE056066	1.22	0.66	7.7	0.0388	AW065996	0.72	1.18	7.0	0.0458
AW787742	1.22	0.72	7.7	0.0395	BE056946	1.11	0.67	7.0	0.0459
AW585288	1.67	0.97	7.6	0.0396	AW288821	0.94	1.25	7.0	0.0460
BE012256	1.04	0.64	7.6	0.0396	AI855258	1.22	0.97	6.9	0.0465
AW787753	1.05	0.78	7.6	0.0400	AW256062	0.78	1.41	6.9	0.0471
AW331165	1.04	1.41	7.6	0.0401	AW257939	0.73	1.66	6.8	0.0475
AW330979	0.81	1.40	7.6	0.0402	AI861106	0.89	1.63	6.8	0.0477
AW231385	0.89	1.41	7.6	0.0402	AI948401	0.85	1.34	6.8	0.0478
AW313219	1.64	1.05	7.6	0.0403	AW621115	0.88	1.54	6.8	0.0481
AW424433	0.75	1.38	7.5	0.0404	AW400101	1.13	0.88	6.8	0.0483
BE012222	1.08	0.70	7.5	0.0405	SP4	0.81	1.87	6.7	0.0486
BE056196	1.22	0.34	7.5	0.0406	AW065950	0.88	1.46	6.7	0.0486
AW256065	0.72	1.14	7.5	0.0408	AW267185	0.70	2.11	6.7	0.0495
AW225221	0.55	1.38	7.5	0.0409	AW455686	0.86	1.16	6.6	0.0495
AW256064	0.68	2.21	7.5	0.0410	AW787851	1.28	0.95	6.6	0.0499
MRP15	0.76	1.95	7.5	0.0413	AW181249	0.92	1.89	6.6	0.0501
BE130026	0.85	1.43	7.4	0.0414	AW288845	0.92	1.26	6.6	0.0501
AW202462	1.18	1.63	7.4	0.0414	AW256147	0.81	1.26	6.6	0.0502

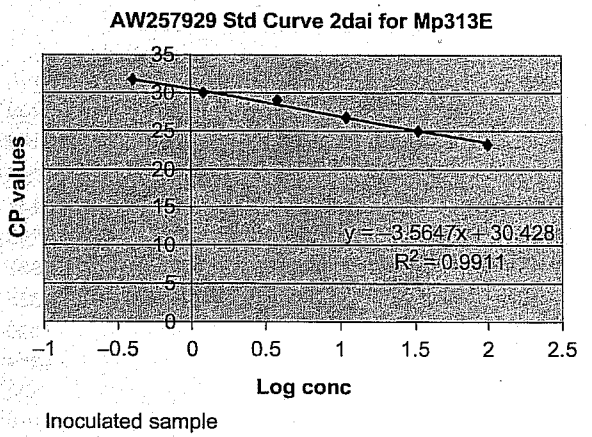
y NCBI accession number (http://www.ncbi.nlm.nig.gov/).
z Statistically significant at $p < 0.05$.

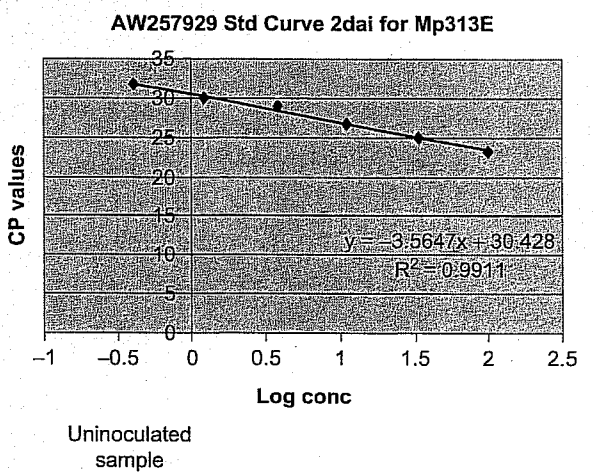
to aflatoxin resistance (Brooks et al., 2005; Davis and Williams, 1999). Using the Maize Genetic and Genomics Database, a comparison of up-regulated genes identified by microarray analysis to gene sequences of QTL identified from Mp313E x B73 and Mp313E x Va35 mapping studies determined that 28 genes have known map locations within the maize genome (Table 1; Figure 2). In addition to the genes that mapped within known QTL, we identified an additional 39 genes that could be mapped to the maize genome (Figure 2). Of the 28 genes mapped to the maize genome QTL, a total of twelve are located on chromosome 1, eight on chromosome 2, nine on chromosome 4, and two on chromosome 6 (Figure 2). The remaining significant genes could not be mapped with the databases available at this time (Table 2).





Figures 3. The real time expression values determined for Beta 5 tubulin chain (AW244904) and NEDD8-like protein RUB2 precursor (AW257929) were normalized to Ubiquitin, a house-keeping gene. Expression values determined by real time were compared with those generated by the microarray analysis. For AW244904 correlation values of 0.995 for inoculated (top panel) and 0.985 for uninoculated (bottom panel) samples were seen for maize line Va35. Similar values were seen for AW257929 (data not shown).





Figures 4. The real time expression values determined for Beta 5 tubulin chain (AW244904) and NEDD8-like protein RUB2 precursor (AW257929) were normalized to Ubiquitin, a house-keeping gene. Expression values determined by real time were compared with those generated by the microarray analysis. For AW257929 correlation values of 0.991 for both inoculated (top panel) and uninoculated (bottom panel) samples were seen for maize line Mp313E. Similar values were seen for AW244904 (data not shown).

qRT-PCR validation

Two genes that mapped within aflatoxin-resistant QTL were selected for quantitative real-time expression analysis (qRT-PCR). Examinations were performed for maize lines Mp313E and Va35 using samples isolated from infected and uninfected samples. The genes selected were Beta 5 tubulin chain (AW244904, up-regulated in Va35) and NEDD8-like protein RUB2 precursor (AW257929, up-regulated in Va35 and Mp313E). These genes were normalized using Ubiquitin. The qRT-PCR expression values showed high correlation to those generated by the microarray analysis (0.985 for all conditions tested) (Figures 3 and 4).

Discussion

QTL studies are undertaken to map regions affecting continuous and quantitative traits, but such studies are time-consuming and the results obtained are often less than accurate due to the size and imprecise resolution of QTL (Li et al., 2005). In contrast, microarrays can allow for the rapid assessment of a large number of genes or cDNA fragments. By mapping candidate genes from cDNA microarray analyses to known QTL data, a more accurate and rapid identification of genes for aflatoxin resistance in maize will result. These important sequences can potentially be used as markers for selecting resistance to *A. flavus* in inbreds generated from resistant crosses as well as having the potential to identify new sources of maize aflatoxin resistance. With the aid of microarray analysis, a total

of 236 genes were detected as significantly increasing in response to *A. flavus* inoculation, 67 of these genes could be mapped within the maize genome (Table 1; Figure 2) and 28 could be mapped within a specific previously identified resistance QTL (Fig. 2) (Brooks et al., 2005; Davis and Williams, 1999). In addition to physically mapping these genes, it is important to place them within a biologically relevant context by examining the biological patterns, based on GO grouping. Two distinctive responses were observed in the biological GO grouping between susceptible (Va35) and resistant (Mp313E) maize lines (Figure 1).

The susceptible maize inbred Va35 showed increased response in genes categorized as biological processes including electron transport, signal transduction, generation of precursor metabolites, response to abiotic and endogenous stimulus, protein metabolism, protein biosynthesis, photosynthesis, response to stress, carbohydrate metabolism, protein modification, and cell cycle (Figure 1). In contrast, the resistant Mp313E maize line showed marked increases in amino acid and derivative metabolism and lipid metabolism (Figure 1).

Although Va35 is considered a susceptible maize line, the biological process profile indicates pathogen recognition and an attempt to respond to the infection. For example, the up-regulation of protein disulfide isomerase (PDI) (accession AW147172) is a clear indication of response to infection. Water-stress inducible protein (AW330570), endochitinase B precursor (AW261292), and a chaperonin (AW066119) are additional genes categorized as response to stress that are also significantly up-regulated in the susceptible line indicating that both biotic and abiotic defenses are active in Va35 (Figure 1). Perturbation of the photosynthetic process is also expected in the susceptible line Va35 since it is well established that pathogen infection leads to both the activation of nuclear defense genes and major changes in primary metabolism of the plant, including reduction in photosynthesis and synthesis of Rubisco (Kombrink and Hahlbrock, 1990; Somssich and Hahlbrock, 1998). This response has been observed in both resistant and susceptible plants, and it has been suggested that this decrease is due to the redirection of resources from growth to defense (Mysore et al., 2003). However, at 48 hours after inoculation, three genes in Va35 (AW331482, AW424439, and AW355894) encoding for chloroplast precursors are significantly up-regulated, not reduced.

The observed recognition of *A. flavus* infection by Va35 but apparent inability to induce sufficient resistance, as compared to Mp313E, may be due to differences in signal transduction pathways between the two lines. One gene up-regulated in Va35 and identified as functioning in transcriptional responses to auxin may shed more light on these signaling differences (Parry and Estelle, 2006). This gene (AW258084) encodes for a transport inhibitor response protein, which is induced in response to endogenous stimulus and acts to inhibit auxin-mediated signaling pathways (Covington and Harmer, 2007). Auxins such as indole-3-acetic acid (IAA) control many crucial plant developmental processes and their regulation appears to be significantly different in the resistant line (Bartel, 1997; Normanly and Bartel, 1999).

Amino acid and derivative metabolism, and lipid metabolism were the two GO categories that contained genes expressed mainly in the resistant line Mp313E (Figure 1). Included in this is BE128894, which is located near but not in QTL 4 and encodes for tryptophan biosynthesis and metabolism. In plants, the tryptophan biosynthesis pathway is essential for synthesis of auxin, phytoalexins, glucosinolates, and both indole and anthranilate-derived alkaloids, and thus plays a direct role in the regulation of plant development and pathogen responses (Radwanski and Last, 1995). It is possible then that the transport inhibitor response protein (AW258084) and the tryptophan biosynthesis gene (BE128894) are both influencing IAA. Other signaling-related genes that are significantly expressed in infected Mp313E are abscisic acid (ABA)-response (AW438153) found on QTL 2 and chalcone synthatase (BE129569) found on QTL 4. Both of these genes are known to function in either general stress response or as signaling genes (Cui et al., 1996; Flors et al., 2005).

The study shows that both Va35 and Mp313E recognize and respond to *A. flavus* infection. The resistant line Mp313E is increasing ABA and auxin signaling, while Va35 appears to be reducing auxin signaling. Additionally, Va35 maintains a higher expression of carbohydrate-related genes. These results may suggest that Va35 up-regulates defense genes but is unable to shift its metabolism efficiently enough to effectively deal with *A. flavus* infection. Moreover, the observed lack of up-regulated defense genes in Mp313E may be a result of either post-transcriptional regulation occurring or induction of genes that are not identified by these arrays.

Our examinations of these two maize lines have identified multiple candidate genes that may be involved in resistance to *A. flavus* infection. A total of 236 genes were found to be significant, and 67 of these could be directly mapped to the maize chromosomes, including 28 located in known QTL associated with resistance. In addition to allowing a better understanding of maize response to fungal infection, these genes are being developed into markers for use in selective breeding of *A. flavus*–resistant maize lines.

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